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Cloning and Characterization of a Gene Involved in Regulation of Sporulation and Cell Division of *Streptomyces griseus*

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A mutant of *Streptomyces griseus*, NY5, differs from the parent in sporulating when grown in a nutrient rich growth medium. Cloned genes of the wildtype organism in the multicopy plasmid pIJ702 transformed into the NY5 mutant cells resulted in converting the mutant phenotype of sporulating in rich medium to the parental genotype of not sporulating in rich medium. A 1.5 kb DNA fragment was sequenced and found to contain two open reading frame gene regions. Restriction deletion mapping and subcloning revealed a gene which when transformed in high copy number into *S. griseus* wildtype, mutant NY5 and *S. lividans* resulted in suppression of sporulation and fragmented cell growth. The gene, named *ssg*A (DDJB/EMBL/GenBank accession no. is D50051), encodes a 145 amino acid protein with a calculated size of 15.8 kDa. The predicted protein has a strong negative charge and shows no significant sequence homology to known proteins. Southern analysis detected regions in *S. coelicolor* and *S. lividans* DNA homologous to *ssg*A.

Streptomycetes are Gram-positive bacteria that grow vegetatively as multinuclear branched substrate mycelia that contain occasional cross walls. Reproduction occurs by the synchronous and regularly spaced septation of specialized aerial hyphae to from uninucleate dormant spores[1].

Most *Streptomyces* species sporulate only during growth on solid media, presumably in response to nutrient depletion signals[1-3]. This point has not been rigidly proven, however. Sporulation in *Streptomyces* is a complicated process involving expression of genes that are both temporally and spatially regulated[1,2]. Much of our understanding of sporulation of streptomycetes has been obtained from genetic studies of *S. coelicolor* grown on solid media. Two classes of sporulation-defective mutants have been analyzed in this organism. One is a class of smooth colony surface (*bld*) mutants which are unable to complete the first stage of sporulation and do not form aerial hyphae or spores[4]. The other class, white (*whi*) mutants, are defective at various stages in aerial mycelium development that do not form mature spores[5]. Recent molecular studies of the *bld*A gene which codes for a rare leucyl-tRNA codon[6] and the *whi*G gene coding for an RNA polymerase σ factor[7] have revealed that the expression of genes involved in the morphological development of *S. coelicolor* can be controlled at both transcriptional and translational levels.

Studies of sporulation of streptomycetes are complicated by problems inherent in their growth on solid surfaces. Sporulation is far from synchronous. At any time, the culture is a mixture of young and senescent vegetative mycelia and hyphae in various stages of the sporulation process. One species, *S. griseus*, sporulates somewhat synchronously in a short time period when cultured in shaken submerged growth media[8,9]. Thus, this organism has advantages for physiological and genetic analyses of sporulation. Some physiological studies have been described[3,9,10]. Genetic and molecular studies of sporulation of *S. griseus* have begun. Babcock and Kendrick[11,12] character-

ized a sporulation gene that converts one class of non-sporulating *bld* mutants to the sporulation phenotype. The gene was found to express two polypeptide products from temporally regulated nested open reading frames having identical C-termini, but different N-termini. The sporulation gene contains a rare TTA codon of leucine, indicating that expression of the gene products is dependent upon a gene related to the *bld*A gene of *S. coelicolor*.

S. griseus strain NRRL B2682 sporulates with a degree of synchrony at a programmed time of 26~30 h when grown in a semi- defined liquid medium[8]. The organism grows profusely as branched mycelia, but does not sporulate when 0.5~1.0% or more of casein hydrolysate or yeast extract are added to the medium. The mechanism of this repression of sporulation, which involves the action of specific amino acids, is unknown. We have approached this question genetically. Mutants of *S. griseus* that sporulate in media containing high levels of casein hydrolysate and yeast extract were isolated[13]. A gene library of parental DNA constructed in a multicopy plasmid vector was transformed into the mutant cells. Six different DNA fragments that convert the mutant phenotype (ability to sporulate in rich growth media) to the parental phenotype (not sporulating in rich growth media) were detected. It is important to point out that the mutants used in this study possess normal sporulation genes and so are quite different from the sporulation defective mutants of *Streptomyces* studied by others. The mutants may be derepressed in some process involving negative regulation of sporulation by components of yeast extract and casein hydrolysate.

We report in this communication the characterization of a gene (*ssg*A) encoded in one of the cloned DNA fragments. The gene encodes a small polypeptide of 145 amino acid residues which when transformed into one of the mutants, NY5, represses the ability to sporulate during growth in rich media. The gene also causes the cells to grow as fragmented rather than mycelial cells. The gene suppresses sporulation and effects fragmented growth when transformed into parental *S. griseus* and into *S. lividans*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media: *S. griseus* strain NRRL B2682 was obtained from the Northern Regional Research Laboratory (Peoria, IL). Mutant NY5 was derived from strain B2682 by *N*-methyl-*N'*-nitro-*N*-nitorosoguanidine (NTG) mutagenesis. This mutant sporulates in both submerged and agar surface cultures in the presence of high levels (1%) of casein hydrolysate and yeast extract where the wildtype strain does not sporulate[13]. *S. lividans* 66 and *S. coelicolor* A3(2) strain M145 were obtained from the John Innes Institute (Norwich, Great Britain). *Escherichia coli* strain DH5α [*sup*E44 (Δ*lac*U169 Ø80*lacZ* ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) was used for cloning experiments.

The high copy number *Streptomyces* plasmid pIJ702[14] was used as a vector for subcloning of a 4.1 kilobase (kb) fragment of genomic DNA. The resultant plasmid, pNY22, confers the wildtype phenotype (non-sporulating in rich media) upon mutant NY5 (sporulating in rich media) strains of *S. griseus*[13]. The *E. coli* vector used was pGEM-3Zf(-) (Promega Corporation, Madison, WI).

Streptomyces cultures were grown in DM1 medium, DMCY (DM1 supplemented with 1% each of casein hydrolysate and yeast extract)[13], trypticase soy broth (TSB) [BBL Microbiology Systems, Cockeysville, MD], SpRM[12], SpRR (SpRM containing 1% each of casein hydrolysate and yeast extract), and R2YE[15]. Solid media contained 1.5% agar. Cultures of *Streptomyces* were cultivated at 30°C on a rotary shaker at 220 rpm in test tubes (10 ml me-

dium/25 ml tube) containing one fifth volume of 4 mm diameter glass beads or in flasks (100 ml medium/500 ml flask) containing a coiled wire spring. For preparation of protoplasts or genomic DNA, the cultures were grown in TSB supplemented with 10% sucrose and 5 mM $MgCl_2$ in the presence of 0.5% (*S. griseus* and *S. coelicolor*) or 0.1% (*S. lividans*) glycine. For plasmid preparation, *Streptomyces* transformants were grown in DMCY. Thiostrepton (E. R. Squibb and Sons, Princeton, NJ) was added to solid or liquid medium at a final concentration of 20 μg per ml or 10 μg per ml, respectively, for growth and selection of thiostrepton resistant transformants. *E. coli* was grown with shaking at 37°C in Luria broth (LB) or on Luria agar (LA)[16]. When necessary, 0.5% glucose was added to LB (LBG) or LA (LAG).

DNA isolation, transformation, and restriction analysis: Total DNA from *Streptomyces* was prepared as described by Hintermann *et al.*[17] and further purified by centrifugation to equilibrium in a CsCl-ethidium bromide density gradient[16]. Plasmid DNA from *Streptomyces* and *E. coli* was isolated following the method of Kieser[18]. For DNA sequencing and preparation of probes, the plasmid DNA was further purified by centrifugation to equilibrium in a CsCl-ethidium bromide density gradient.

Streptomyces strains were transformed as described by Babcock and Kendrick[11], except that 10 μg of sonicated calf thymus DNA (Sigma Chemical Co., St. Louis, MO) and three volumes of 45% polyethylene glycol 1000 (Sigma) solution was added to the transformation mixture. After transformation, protoplasts were plated on SpRM, SpRR (*S. griseus*), or R2YE (*S. lividans*), and allowed to regenerate for 15~20 h at 30°C. A 1 ml solution of 10% sucrose and 0.2 mg per ml of thiostrepton was then poured over the agar surface. The cultures were incubated for a further 3~4 d. Transformation of *E. coli* was done according to standard procedures[16]. Transformants were selected on plates containing ampicillin (50 μg per ml). When required, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 μg per ml) and isopropyl-β-D-thiogalactopyranside (IPTG) (0.5 mM) were added to LA or LAG.

DNA manipulations: Restriction endonucleases, T_4DNA ligase, the Klenow fragment of DNA polymerase I, and T_4DNA polymerase were obtained from Stratagene (LaJolla, CA). Calf intestinal alkaline phosphatase and deoxynucleotide stocks were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Buffer and reaction conditions were those specified by the vendors. Restriction fragments were separated by horizontal agarose gel electrophoresis[16]. DNA fragments for subcloning and for use as probes were obtained by isolation of the relevant restriction fragments from agarose gels with a QIAEX kit (Qiagen Inc., Chatsworth, CA). DNA fragments were radiolabeled by using a PRIME-IT random primer labeling kit (Stratagene) and [α-^{32}P] dATP (spec. act. 3000 Ci/mmole) (Du Pont, Boston, MA).

Plasmid construction: 1) Subclones in pIJ702 (Fig. 2). pNY221 was constructed by religating *Bgl*II digests of pNY22 into pIJ702 under dilute conditions. pNY222 and pNY222R, differing in the insertional orientations, were constructed by ligating a 3.4 kb *Bam*HI-*Bgl*II insert fragment from pNY22 to the *Bgl*II-digested pIJ702 followed by treatment with alkaline phosphatase. The 1.5 kb *Bam*HI-*Pst*I fragment or 1.8 kb *Bgl*II-*Pst*I fragment of the insert DNA from pNY22 was ligated to the *Bgl*II-*Pst*I large fragment of pIJ702 to yield pNY223 or pNY224, respectively. For construction of pNY225, the 1.5 kb *Bam*HI-*Pst*I fragment was blunt-ended by filling in the 5' and 3' overhangs using T_4DNA polymerase and then ligated to the *Bgl*II digested pIJ702 that was blunt-ended by using the Klenow fragment of DNA polymerase I. For construction of pNY226 to pNY230, the 1.5 kb *Bam*HI-*Pst*I fragment (containing the *ssg*A gene) was first

purified by gel electrophoresis. The vector pIJ702 was digested with *Bgl*II and blunt-ended as above. The 1280 bp fragment or 1109 bp fragment obtained from the 1.5 kb fragment by digestion with *Cla*I or *Aha*II was blunt-ended and ligated to the linearized pIJ702 to yield pNY226 or pNY227, respectively. pNY228 was constructed by ligating the 981 bp fragment obtained from the 1.5 kb fragment by digestion with *Hinc*II to the linearized pIJ702. The 860 bp and 607 bp fragments obtained from the 1.5 kb fragment by digestion with *Sac*II and *Sma*I were blunt-ended and ligated separately to the linearized pIJ702 to yield pNY229 and pNY230, respectively. The ligation mixtures were transformed directly into *S. griseus* strain NY5. Plasmid minipreparations, selected from thiostrepton resistance and melanin non-producing transformants, were examined for the presence of the appropriate fragment and its insertional orientation by restriction analysis.

2)Subclones in pGYM-3Zf(-). The 1.5 kb *Bam*HI-*Pst*I fragment containing the *ssg*A gene was ligated to similarly digested pGYM-3Zf(-) to yield pUWB. For DNA sequence of the fragment, nested sets of deletions were generated from both sides of the *ssg*A insert in pUWB by the unidirectional exonuclease III procedure of Henikoff[19] with the EXOIII/MUNG BEAN deletion kit (Stratagene). The restriction sites to provide exonuclease-sensitive and -resistant ends were *Bam*HI and *Kpn*I at one side or *Cla*I (approximately 200 bp inside the *ssg*A fragment from the polylinker region of the vector; Fig. 3) and *Pst*I at the other site, respectively.

3)Gene fusion plasmids. Constructs pLac3 and pLac1R: pGEM-3Zf(-) were digested with *Hinc*II (cutting at the 23rd codon of the N-terminal α-peptide encoded by the partial *lac*Z gene) and were then dephosphorylated. A deletion plasmid of pUWB containing the *ssg*A fragment corresponding to nucleotide numbers 1 to 1050 (Fig. 3) was digested with *Sac*I and *Hind*III, cutting at the 27th codon of the *ssg*A polypeptide and at the polylinker region of the vector located to the 3' end of the fragment, respectively. The resulting 610 bp fragment was blunt-ended with T_4DNA polymerase, ligated to the linearized vector, and introduced by transformation into *E. coli* DH5α. Ampicillin resistant transformants were isolated on LAG plates containing ampicillin. Restriction analysis was then performed on the plasmid minipreparations. pLac3 (Fig. 4A) contained the *ssg*A fragment in the correct orientation to produce a fusion protein. pLac1R contained the fragment in the opposite direction. pLLa1: pGEM-3Zf(-) was digested with *Sma*I (cutting at the 29th codon of the α-peptide) and dephosphorylated. pUWB was digested with *Hinf*I and *Hind*III (cutting at the third codon of the *ssg*A polypeptide and at the polylinker region of the vector, respectively), and the resulting 1142 bp fragment was blunt-ended. The fragment was ligated to the linearized vector to yield pLLa1, in order to produce a fusion protein.

SDS-polyacrylamide gel analyses: *E. coli* DH5α containing the plasmid expressing a fusion protein or the control plasmid were grown overnight in LBG. A 1 ml sample of the cultures was inoculated into 10 ml of fresh LBG medium containing ampicillin in the absence or presence of IPTG (0.5 mM), or LB containing ampicillin. The LBG or LB cultures were incubated for 8 h or 12 h, respectively. The cells were harvested, washed with buffered saline (10 mM sodium phosphate buffer, pH 7.0 - 150 mM NaCl) by centrifugation, and then suspended in water at a cell density of about 150 Klett units (red filter). A 10 μl sample of the cell suspension was mixed directly with sample buffer and subjected to 0.1% SDS-12.5% polyacrylamide gel electrophoresis (PAGE)[20]. Protein bands were visualized by the Coomassie brilliant blue staining procedure.

DNA sequence analysis: Nucleotide sequences were determined by the dideoxynucleotide chain termination method using a Seque-

nase version 2.0 kit (United States Biochemical Corporation, Cleveland, OH), alkali-denatured supercoiled plasmid DNA, and [α-^{35}S] dCTP (spec. act. 300-400 Ci/mmole, Du Pont). In sequencing reactions, 7-deaza-dGTP was substituted for dGTP[21,22].

Southern hybridization analysis: Transfer of DNA from agarose gels to nylon membranes (Zeta-Probe; Bio-Rad Laboratories, Richmond, CA) was carried out as described by Smith and Summers[23]. Prehybridization and hybridization was conducted at 70°C in the hybridization solution as described by Virca *et al.*[24]. The filters were washed two times in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0)-1% SDS for 5 min each at room temperature, followed by two 15 min washes each in 2 × SSC at 55°C and one wash in 0.2 × SSC-1% SDS at 65°C.

Computer analysis: The sequence analysis of DNA and protein by computer was carried out using the GCG Package program (Genetics Computer Group Inc., Madison, WI). The deduced sequence of the *ssg*A gene product was compared with the entries in the EMBL and GenBank data bases using the program FASTP[25].

RESULTS

Transformation with pNY22 effects fragmented growth and spore minus phenotypes: Parental cells of *S. griseus* when grown in the complex DMCY medium form long, branched filaments, but do not sporulate. A mutant, NY5, sporulates profusely in the medium. This pattern of growth was not changed when the cultures were transformed with the plasmid pIJ702 (Fig. 1A and C). Transformation of plasmid pNY22 containing a 4.1 kb wildtype genomic insert in pIJ702 (see Fig. 2) into parental cells resulted in the culture growing as fragmented, irregularly shaped rods (Fig. 1B). No spores were produced. The shaken DMCY culture of pNY22 transformed cells grew very well in dispersed manner and reached an absorbance 600 nm of 1.1 at 24 h. Mutant NY5 transformed with pNY22 also grew as fragmented cells and lost the capacity to sporulate (Fig. 1D). Parental and NY5 cells grew as filaments and sporulated in DM1 medium. Transformation with pNY22 caused growth of both in DM1 to become fragmented with no sporulation (data not shown).

These data show that a 4.1 kb genomic DNA insert of pNY22 caused a change in growth pattern from branched filamentous cells, which are multinucleate and non-septated, to fragmented pleomorphic rod-shaped cells. The insert DNA also caused suppression of sporulation. Induction of fragmented growth of parental cells suggests that the 4.1 kb gene in pNY22 DNA encodes a positive regulatory gene which promotes cell septation. Overproduction of the gene product by a gene dosage effect might

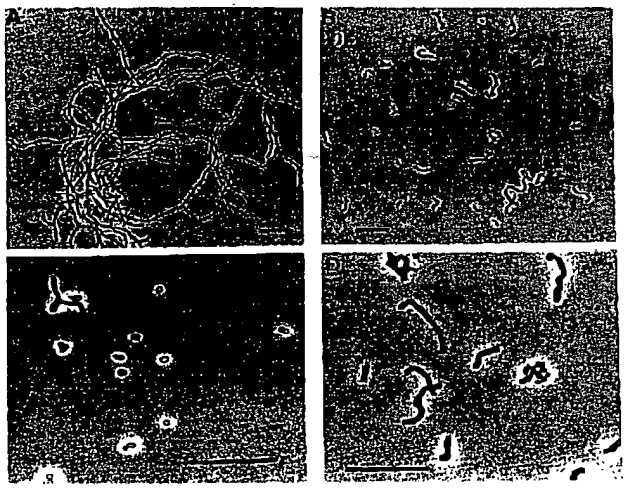
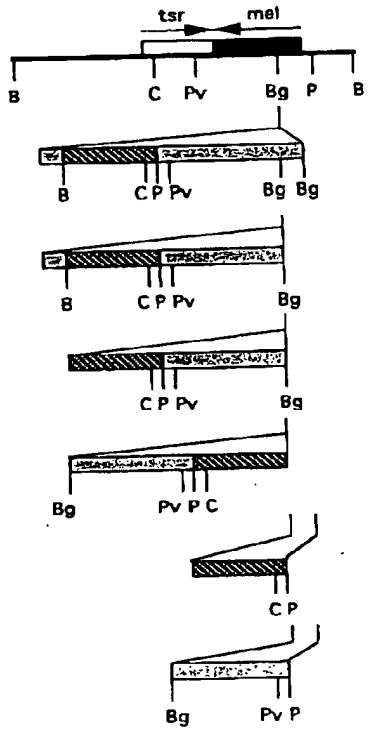


Fig. 1. Phase contrast photomicrographs of parental and NY5 mutant cells following transformation with pIJ702 or pNY22. Photographs taken of cells following 48 h growth in DMCY medium containing thiostrepton. A: parent cells transformed with pIJ702, B: parent cells transformed with pNY22, C: NY5 transformed with pIJ702, D: NY5 transformed with pNY22. Bars represent 10 μm.



Plasmid (size, kb)	Insert size (kb)	Phenotype of NY5 transformants: Sporulation DMCY	Sporulation DM1	Fragmented growth DMCY	Fragmented growth DM1
pIJ702 (5.8)	0	+	+	No	No
pNY22 (9.9)	4.1	-	-	Yes	Yes
pNY221 (9.5)	3.7	-	-	Yes	Yes
pNY222 (9.2)	3.3	-	-	Yes	Yes
pNY222R (9.2)	3.3	-	-	Yes	Yes
pNY223 (6.9)	1.5	-	-	Yes	Yes
pNY224 (7.2)	1.8	+	+	No	No

Fig. 2. Minimum size region of genomic DNA insert of pNY22 conferring suppression of sporulation and fragmented growth phenotypes on NY5 cells. Restriction enzyme generated fragments of the 4.1 kb genomic DNA insert of pNY22 were transformed via pIJ702 into mutant NY5 cells. Transformants were grown for 7 d on DMCY and DM1 agar containing thiostrepton and observed microscopically for spores (+ spores present, - no spores present) and for fragmented growth in submerged culture. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; P, *Pst*I; Pv, *Pvu*II. B/Bg represents the junction formed by isozysomeric ligation which is not cleaved by either enzyme.

cause the fragmented growth.

Minimum size of pNY22 conferring suppression of sporulation and fragmented growth phenotypes: The 4.1 kb genomic DNA insert of pNY22 was hydrolyzed by restriction enzymes and the fragments were transformed using pIJ702 into mutant NY5 cells. The plasmid constructs and their effects on growth and sporulation of NY5 are shown in Fig. 2. Introduction of all the subclones, except pNY224, conferred the fragmented growth and suppression of sporulation phenotypes on NY5 cells. This was true for growth in both DM1 and DMCY media. These data show clearly that a 1.5 kb region between *Bam*HI and *Pst*I located to the left half of the insert DNA in pNY223 confers both phenotypes. This DNA segment appears to contain its own transcriptional control domain since its effect was not affected by insertional orientations (pNY222R versus pNY222 and pNY223).

The effect of transforming the six subclone plasmids into *S. lividans* was tested. All of the subclones, except pNY224, suppressed sporula-

tion and caused fragmented growth in R2YE medium (data not shown). The organism sporulated and grew as filamentous cells when transformed with pIJ702 and pNY224.

The gene encoded by the 1.5 kb fragment of parental *S. griseus* DNA in pNY223 is named *ssg*A because of its strong suppressive effect on sporulation.

Nucleotide sequence and localization of ssgA gene: The nucleotide sequence of the entire 1.5 kb *Bam*HI-*Pst*I fragment of pNY223 was determined using plasmid pUWB containing the fragment in pGEM-3Zf(-). Nested deletions of pUWB were constructed from both junction sites of the insert DNA and both strands were completely sequenced. The results, Fig. 3, show that two possible open reading frames were found in the same strand of the complete nucleotide sequence. The computer analysis used, the Codon Preference + program, recognizes protein coding sequences by virtue of similarity of their codon usage to a codon frequency table[26] or the third position GC bias in each codon[27]. The *Streptomyces* codon frequency table (stm 7074.cod) kindly provided by Dr. C. R. Hutchinson was used for this analysis. ORF1 codes for a polypeptide chain of 145 amino acids (aa) starting with an ATG initiation codon at nucleotide 365 and terminating with a TGA stop codon at nucleotide 802 (Fig. 3). ORF2 codes for a polypeptide chain of 240 amino acids starting with an ATG initiation codon at nucleotide 573 and terminating with a TAG stop codon at 1295. The frequency of rare codon usage characteristic of *Streptomyces* was approximately 12% (18/145) and 21% (51/240) in ORF1 and ORF2, respectively. The two genes overlapped partially, but were translated in different frames.

The location of the *ssg*A gene was determined by further subcloning of different fragments of the 1.5 kb *Bam*HI-*Pst*I DNA segment into pIJ702 followed by transformation of NY5 cells. The transformants were observed for sup-

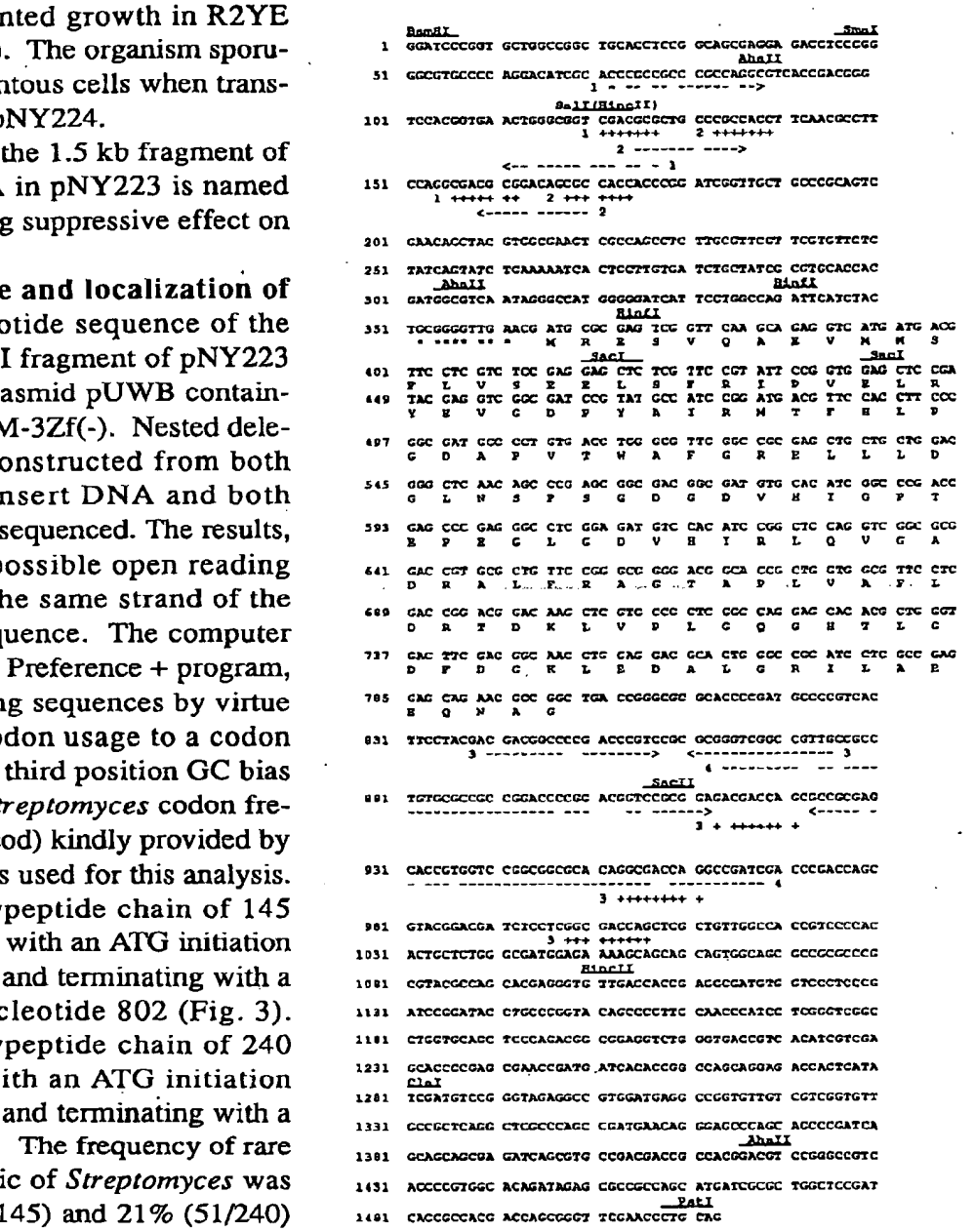
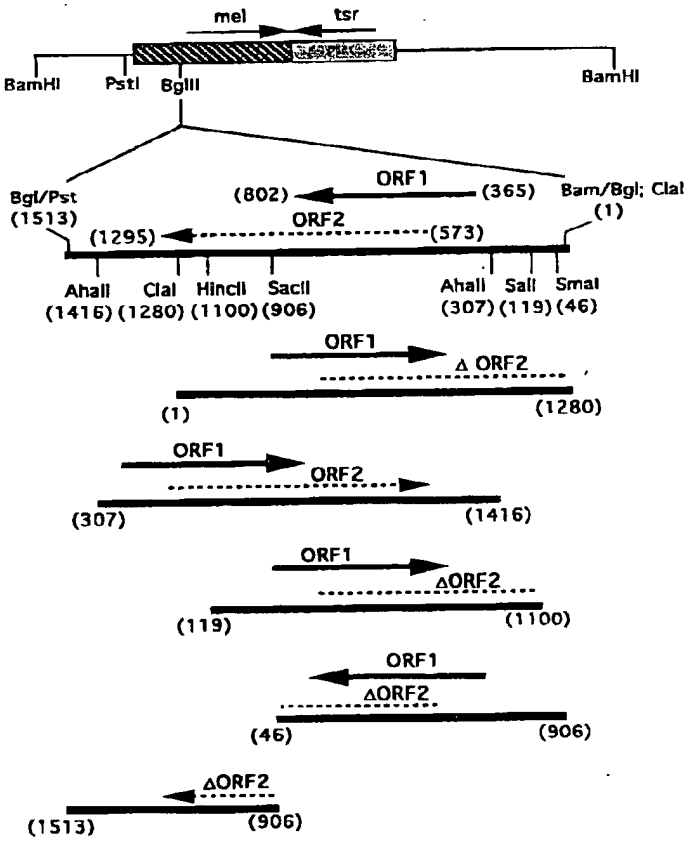


Fig. 3. Nucleotide sequence of the 1.5 kb *Bam*HI-*Pst*I fragment of pNY22 and the deduced amino acid sequence of the *ssg*A gene product. The asterisks denote the putative ribosome binding site. The plus sings marked 1, 2, and 3 indicate direct repeats. Four long inverted repeat sequences are shown by the broken lines with arrowheads. Only relevant restriction sites are shown. The nucleotides are numbered starting with the *Bam*HI site.

pression of sporulation and fragmented growth in both DM1 and DMCY media. The plasmid constructs and a summary of their effects on NY5 are shown in Fig. 4. Plasmid pNY225 containing intact ORF1 and ORF2, and plasmids pNY226, pNY228, and pNY229 all containing intact ORF1 but truncated ORF2 with different sized C-terminal fragments removed, suppressed sporulation and conferred fragmented growth. pNY227 with portions of the 5'- and 3'-terminal DNA removed by *Aha*II digestion, but still containing ORF1 and 2, transformed NY5 to the spore-minus fragmented growth phenotype when grown on DMCY medium. However, the transformant grew as filamentous mycelia and sporulated in DM1 medium. The number of spores was less and their appearance occurred later than was observed with control pIJ702 transformed cells. The pNY230 construct lacking the entire ORF1, but containing



	Phenotype of NY5 transformants			
	Sporulation		Fragmented growth	
	DMCY	DM1	DMCY	DM1
pIJ702	+	+	No	No
pNY225	-	-	Yes	Yes
pNY226	-	-	Yes	Yes
pNY227	-	+	Yes	No
pNY228	-	-	Yes	Yes
pNY229	-	-	Yes	Yes
pNY230	+	+	No	No

Fig. 4. Determination of the open reading frame encoded by the *ssg*A gene. The 1.5 kb sequenced DNA fragment (Fig. 3.) was used to construct the illustrated plasmids. The different deletions (black thick bar) of the fragment were ligated into the *Bgl*II site of pIJ702 at the indicated orientations. Solid (marked ORF1) and broken (marked ORF2) lines with arrowheads (showing the direction of translation) represent possible open reading frames. The sporulation and fragmented growth phenotypes were determined using the NY5 transformant of the indicated plasmid.

the C-terminal portion of ORF2 did not have any affect when transformed into NY5. These results suggest that the *ssg*A gene, including its own transcriptional control region, is contained in the 787 bp *Sal*I-*Sac*II fragment (nt 119~906). This gene is responsible for both the suppression of sporulation and abnormal fragmented growth phenotypes in the mutant strain NY5 when grown in DM1 and DMCY media.

The *ssg*A gene product, ORF1, has a high GC content (68%) and typical *Streptomyces* codon usage (Fig. 3). Codons with G or C in the third position are very frequent (89%), as has been described for other *Streptomyces* genes[15]. A region located 4 to 13 nucleotides upstream of the ATG codon is a potential ribosome-binding site (RBS)[27]. Two imperfect 17 and 12 base pair inverted repeats and also two perfect direct repeats (7 base pairs each) are present within a relatively narrow region (from nucleotides 71 to 174) further upstream from the RBS. The upstream 183 bp *Sal*I-*Aha*II fragment (from nt 121 to 304), which includes part of these repeats, appears to be very important for *ssg*A gene expression. Both pNY227 and pNY228 contain a complete ORF1 including the putative RBS. The pNY228 construct, containing the *Sal*I-*Aha*II fragment, caused suppression of sporulation and fragmented growth in both DM1 and DMCY media. Transformants of the pNY227 plasmid, which lack the *Sal*I-*Aha*II fragment, caused suppression of sporulation and fragmented growth only in DMCY medium. This effect of pNY227 might be the result of expression of the *ssg*A gene from the tyrosinase gene (*mel*) promoter located next to the *Bgl*II site of pIJ702. Gene expression from the *mel* promoter is expected to increase significantly because of the rich nutrients in DMCY[14]. It is likely, therefore, that the intact *ssg*A promoter is not present in pNY227.

Two inverted repeats and a direct repeat are contained with a narrow region (nt 835 to 970) downstream from the translation stop codon. An imperfect long inverted repeat that is capable of forming a very stable stem and loop structure (G = -69.3 Kcal) in the region could act as a transcriptional terminator. The presence of the DNA region forming possible strong secondary structure in the putative ORF2 suggests that ORF2 is unlikely to be translated.

The amino acid sequence deduced from the DNA sequence of ORF1 indicates a similar number of hydrophobic amino acids and hydrophilic amino acids (63 and 59, respectively). The *ssg*A polypeptide is predicted to be relatively acidic, containing 27 strongly acidic amino acids and 15 strongly basic residues. The calculated isoelectric point is approximately 4.3. A Kyte-Doolittle algorithm analysis showed the protein to be highly hydrophilic with two hydrophobic regions (Fig. 5.). A secondary structure analysis using the GCG Peptide structure program predicts that the protein is cytoplasmic with no membrane or DNA binding domains. Antibodies prepared against the protein confirmed in Western blot analysis that the protein is located in the cytosol (Kawamoto, unpublished observation). No extensive sequence similarity between the *ssg*A gene product and any other known protein coding

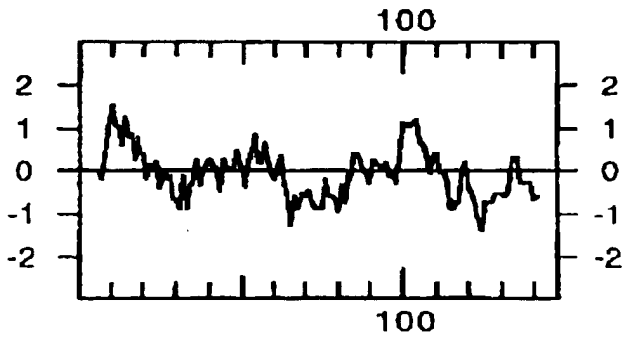


Fig. 5. Hydrophobicity plot of *ssg*A protein. The program DNA Strider was used to plot hydrophobicity using the Kyte-Doolittle algorithm. Hydrophobic regions above the axis, hydrophilic regions below. Axis: amino acid number.

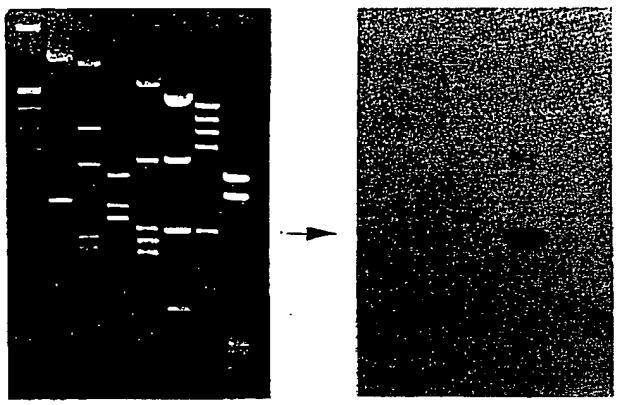


Fig. 6. Southern hybridization analysis of homology of *ssg*A gene to recombinant plasmids which confer the spore-minus phenotype when transformed into NY5 cells. (A) Ethidium bromide stained fragments separated by electrophoresis through 1% agarose gel. Lane 1, fragment size markers (21.2, 16.4, 9.6, 7.9, 7.0, 5.1, 4.3, 3.5, 2.8, 2.7, 2.0, 1.9, 1.6, 1.5, 0.95, 0.83, 0.60, and 0.53 kb); lane 2, *Bam*HI and *Pst*I digest of pSY12; lane3, *Bam*HI and *Bgl*III digest of pSY31; lane 4, *Sal*I digest of pNY11; lane 5, *Bam*HI digest of pNY14; lane 6, *Bam*HI and *Pst*I digest of pNY22; lane 7, *Bam*HI and *Bgl*II digest of pNY33; lane 8, *Sal*I digest of pIJ702. (B) Probe using the *Bam*HI-*Pst*I 1.5 kb fragment from pNY22 for detecting homology to *ssg*A. Photograph of autoradiograph exposed at -80°C for 3 h.

sequence was detected in the GenBank and EMBL data bases.

Southern hybridization studies: Our previous study reported five independently derived plasmids which, when transformed into mutant NY5 cells. caused a loss of sporulation of the cells during growth in DMCY medium[13]. Restriction analyses of these five plasmids, with inserts of genomic DNA of 1.8~12.1 kb, and pNY22 containing the *ssg*A gene suggest that the inserts were derived from different regions of the parent genome. In order to determine if the *ssg*A gene was encoded in any of these plasmids, single or double-restriction digests of the recombinant plasmids and the vector pIJ702 were hybridized with the 1.5 kb *Bam*HI-*Pst*I fragment of pNY22 containing the *ssg*A gene as a probe. The results of these analyses, Fig. 6A and B, show that a single hybridization signal was detected only in the corresponding fragment of pNY22. This indicates that the *ssg*A gene does not have any sequence homology to the cloned DNA fragments contained in the other five plasmids. This result is expected from the restriction analyses[13].

An experiment was designed to determine if the *ssg*A gene is present in *S. lividans* and *S. coelicolor*. Total DNA of the parental *S. griseus* strain and its mutant NY5 and of *S. lividans* and *S. coelicolor* was digested with either *Bam*HI and *Bgl*II or *Bam*HI and *Pst*I. The DNA fragments were hybridized with a probe of pUWB containing the 906 base pair *Bam*HI-*Sac*II *ssg*A gene fragment. The results are shown in Fig. 7. The DNA of parental and the NY5 mutant of *S. griseus* showed single *ssg*A hybridization bands of approximately 5.4 kb for the *Bam*HI-*Bgl*II digests (lanes 3 and 7) and 3.0 kb for the *Bam*HI-*Pst*I digests (lanes 4 and 6). The *ssg*A gene showed hybridization to two bands, approximately 3.0 and 1.4 kb, of *Bam*HI-*Bgl*II digested DNA in *S. lividans*.(lane 8). The gene also hybridized to two bands of 1.9 and 1.1 kb with *Bam*HI-*Pst*I digested DNA (lane 9) in the organism. *S. coelicolor* digested with *Bam*HI-*Bgl*II showed two bands of hybridization with sizes 2.7 and 1.2 kb (lane 10), and 1.5 and 0.9 kb with the *Bam*HI-*Pst*I digest (lane 11). The strength of the hybridization signal was much lower in *S. lividans* and *S. coelicolor* than for *S. griseus* with the high stringency hybridization conditions employed in these experiments. These data suggest that the *ssg*A gene is present in *S. coelicolor* and *S. lividans*, but in a lesser homology to that of *S. griseus*.

The sizes of the hybridization band obtained with the total DNA digest of *S. griseus* with *Bam*HI and *Bgl*II (5.4 kb) or with *Bam*HI and *Pst*I (3.0 kb) were not the sizes of 3.4 kb or 1.5

map of the insert DNA of pNY22 (Fig. 2). This observation suggests that the genomic insert DNA had sustained some rearrangements during the course of the cloning procedures. In order to determine if this had occurred, further Southern blotting analyses were made to deduce the restriction map of the *ssg*A region of the genome. Different fragments of the insert DNA in pNY22 were used as probes with total DNA digests obtained with several restriction enzymes. Alignment of the restriction maps of the pNY22 insert DNA and the *ssg*A region on the parent genome indicates that the *Bam*HI site located near the left end of the pNY22 insert DNA is missing in the corresponding region of the parent genome (data not shown). Colinearity of the restriction map of the pNY22 insert DNA and the *S. griseus* genome is present from this *Bam*HI site (missing in the genome) to the *Bgl*II site located at the right end of the pNY22 insert. The results suggest that an approximate 300 base pair region located at the left end of the pNY22 insert DNA could have originated from an unrelated genomic DNA region which had been ligated to the 3.8 kb *Sau*3AI-*Bgl*II fragment of the *ssg*A region. This generated a new *Bam*HI site at the junction site during the course of cloning that employed partial *Sau*3AI digests of the parental genomic DNA.

Expression of LacZ-SsgA fusion proteins in *E. coli*: In order to confirm the putative *ssg*A ORF1 deduced by the DNA sequence and deletion analyses, fusion genes between an N-terminal α-peptide-encoding *lac*Z DNA and the putative ORF1 were constructed. The truncated *ssg*A DNA lacking the 3 N-terminal amino acids or 27 amino acids of ORF1 was ligated in frame downstream of the a-peptide in pGEM-3Zf(-). The structure of the fusion genes is illustrated in Fig. 8A. When *E. coli* DH5α transformants were selected on LA media containing ampicillin and X-gal either in the presence or absence of IPTG, white recombinant colonies were observed. The 12 clones that were tested

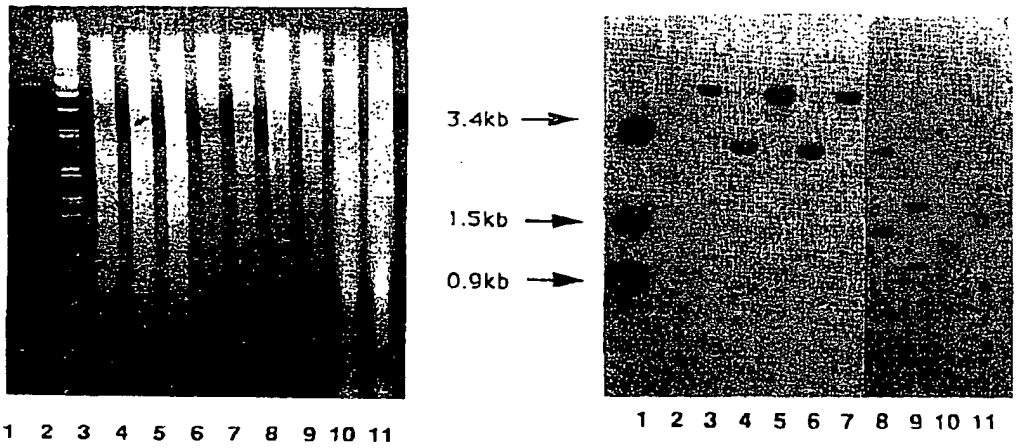


Fig. 7. Homology between the *ssg*A gene and genomic DNA of parental and mutant NY5 of *S. griseus*, *S. lividans*, and *S. coelicolor*. (A) Ethidium bromide stained fragments separated by electrophoresis through 1% agarose gel. Total DNA of parental *S. griseus* (lanes 3, 4, 5) and mutant NY5 (lanes 6, 7), *S. lividans* (lanes 8, 9), and *S. coelicolor* (lanes 10, 11) were digested with either *Bam*HI and *Bgl*II (lanes 3, 5, 7, 8, 10) or *Bam*HI and *Pst*I (lanes 4, 6, 9, 11). Lane 1 contains three fragments of the *ssg*A gene as a positive control: 3.4 kb *Bam*HI-*Bgl*II, 1.5kb *Bam*HI-*Pst*I, 0.9 kb *Bam*HI-*Sac*I. Size standards, lane 2, as in Fig. 6. (B) Probe using the *Bam*HI-*Pst*I fragment from pNY22 for detection of homology to *ssg*A. Photograph of autoradiograph exposed at -80°C for 24 h (lanes 1~7) or 48 h (lanes 8~11).

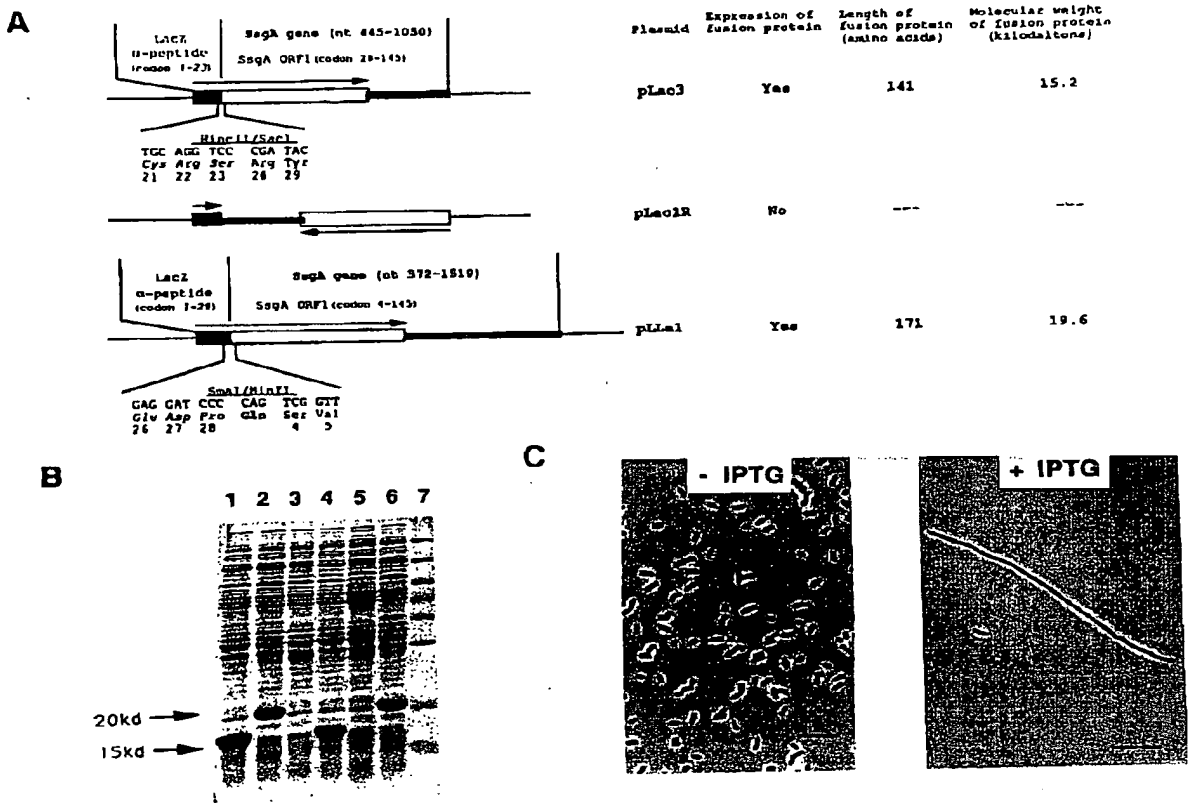


Fig. 8. Expression of the *lacZ-ssgA* fusion proteins in *E. coli* DH5α. (A) Structure of the fusion genes between *lacZ* and *ssgA* constructed in *E. coli* vector pGEM-3Zf(-). (B) SDS-PAGE analysis of the fusion proteins. *E. coli* DH5α cells harboring pLac3 (lanes 1, 3, and 4), pLac1R (lane 5), and pLLa1 (lanes 2 and 6) were grown overnight in LB containing ampicillin (lanes 1 and 2) or grown for 8 h in LBG containing ampicillin without IPTG (lane 3) and in the presence of 0.5 mM IPTG (lanes 4–6). Lane 7 shows size standard markers (97.4, 66.2, 55, 42.7, 40.0, 31.0, 21.5, and 14.4 kd). Arrows indicate the fusion protein bands. (C) Photomicrographs of pLac3 transformant cells of *E. coli* grown for 8 h in LBG containing ampicillin in the absence (left) or presence (right) of IPTG. The bars represent 10 μm.

all contained plasmids with the *ssgA* fragment in the opposite orientation and did not produce a fusion protein. The *E. coli* DH5α cells do not have a *lacI*^{q} mutation overproducing the *lac* repressor[28]. The cells transformed with pGEM-3Zf(-) formed blue colonies on LA medium containing ampicillin and X-gal without IPTG because of the absence of enough *lac* repressor to completely repress α-peptide expression from the *lac* promoter of the high copy number plasmid. We expected that the *lacZ-ssgA* fusion protein would be substantially expressed in the medium, even in the absence of IPTG, and that overexpression of the fusion proteins could be detrimental to growth of the cells. Therefore, transformants were selected on LAG media containing ampicillin. Blue color development by the pGEM-3Zf(-) transformant colonies was completely suppressed in this medium, probably due to strong catabolite repression by glucose. Two different fusion gene plasmid, designated as pLac3 and pLLa1 (Fig. 8A), were obtained. pLac3 is expected to direct the synthesis of a fusion protein of 15.2 kd (141 amino

acids) that consists of the N-terminal 23 amino acid residues of α-peptide and C-terminal 118 amino acid residues of the *ssg*A ORF. pLLa1 is expected to direct the synthesis of a fusion protein of 19.6 kd (171 amino acids) that consists of the N-terminal 28 amino acid residues of α-peptide, a glutamine residue generated in the construction, and the C-terminal 142 amino acid residues of the *ssg*A ORF. The protein fusion sites of the plasmid were confirmed to be correct by DNA sequencing analysis. Cells of *E. coli* DH5α were transformed with the three plasmids and grown overnight in LB medium and for 8 h in LBG medium in the presence and absence of IPTG. The results of SDS-PAGE analyses of the cell proteins are shown in Fig. 8B. Both pLac3 and pLLa1 transformants accumulated large amounts of the fusion proteins when grown in LBG medium in the presence of IPTG (lane 4 and 6). A transformant containing the control plasmid pLac1R, in which the *ssg*A DNA was inserted in the opposite direction, did not form the fusion protein when grown in the medium (lane 5). The sizes of the fused proteins detected by SDS-PAGE were in good agreement with those expected (see above). The transformants also accumulated a comparable amount of the fused proteins after overnight incubation in LB without IPTG. These data confirm the putative *ssg*A ORF1. Long, filamentous cells which contain multiple phase dark inclusion bodies were formed during growth of the cells for 8 h in LBG medium containing IPTG (Fig. 8C). Cells grown without IPTG did not form inclusion bodies and were normal in size and morphology. Similar morphology, with inclusions, was also observed in 18~24 h cultures growing in LB medium (data not shown).

DISCUSSION

Sporulation of many species of *Streptomyces* is repressed during growth in media with high levels of complex organic substrates such as yeast extract or casein hydrolysate. This effect on sporulation was attributed to three individual amino acids, histidine, glutamic acid, and aspartic acid for *S. viridochromogenes*[29] and to one amino acid, valine, for *S. griseus*[8]. Sporulation of *S. griseus* was repressed by valine during growth both in liquid and on solid media. The mechanism of this repression of sporulation is not known. We previously described two mutants of *S. griseus* that acquired the ability to sporulate in complex media where the parent cells do not sporulate[13]. Transformation of six different genomic DNA fragments from the parent to the mutant cells caused the mutants to reacquire the original phenotype of not sporulating in the complex media. One of these fragments containing the gene *ssg*A also caused the cells to grow in a fragmented rather than mycelial fashion. Transformation of the gene in high copy number into the parent wildtype cells or into another species, *S. lividans*, caused the recipients to become fragmented and non-sporulating during growth in DM1 medium. In this medium, both organisms normally grow as branched mycelia and sporulate. Thus the *ssg*A gene product, a 15.8 kDa acidic protein, acts in two ways; as a positive effector of cell division and as a negative effector of sporulation.

The *ssg*A gene contains no TTA codons, an extremely rare leucyl tRNA codon in *Streptomyces*. This observation and the finding of no sequence homology indicates that *ssg*A is not related to the *bld*A gene that is involved in sporulation and aberrant septum formation in *S. coelicolor*[6] or the analogous gene in *S. griseus*[30]. The *ssg*A gene also no sequence homology to the *whi*G gene that is involved in septum formation in terminal stages of sporulation of *S. coelicolor*[7,31]. The *whi*G gene when transformed into wildtype *S. coelicolor* by a multicopy plasmid caused sporulation to occur on hyphae growing below the solid agar surface and in submerged culture, both processes which

do not normally occur with this organism[31]. The *whi*G gene, which codes for an RNA polymerase sigma factor[7], appears to be a positive regulator of sporulation.

The stimulation of fragmented growth, most likely involving a stimulation of septum formation, raises the possibility that *ssg*A may be related to septum forming gene in bacteria. One possibility is the *fts*Z gene involved in septum formation in *E. coli* and in both the vegetative and sporulating cells of *Bacillus subtilis*[32,33]. The *ssg*A gene shows no nucleotide sequence homology to *fts*Z or to a gene of similar sequence found in *S. griseus*[34].

The *ssg*A gene product, being a small highly acidic protein with no DNA or membrane binding domains, is not likely to be a component of the cell division machinery in the cell membrane. It is possible that *ssg*A is functionally analogous to the *min*E gene of *E. coli* which acts as a topological specific factor in septum formation during cell division[35,36]. The *min*E gene product is a small protein that prevents a division inhibitor coded for by two other genes, *min*C and *min*D, from acting at the midcell septum site while permitting the inhibitor to block septa formation at other sites. Overexpression of *min*E titrates out the inhibitor, allowing cell division at these sites leading to minicells. Analogous overproduction of *ssg*A could react with a division inhibitor in *Streptomyces* and result in the observed fragmented growth. This hypothesis is consistent with the induction of fragmented growth of parental *S. griseus* and *S. lividans* following transformation with *ssg*A carried by a multicopy plasmid. This could also explain the filamentous cells of *E. coli* that resulted from expression at high level of the *ssg*A gene. Function similarity of *ssg*A and *min*E might block action of *min*E, resulting in inhibition of septum formation due to the division inhibitor. If there is function similarity of these two genes, it is not reflected in sequence homology where there is none.

The *ssg*A protein has been purified from the fusion protein overexpressed in *E. coli* and antibodies have been prepared (SK, unpublished results). The time controlled and synchronous sporulation of *S. griseus* will make it possible to study the time course of *ssg*A gene expression and location of its small acidic protein product during mycelial growth and sporulation. We anticipate that the extended studies will yield important information about the connection between sporulation and cell division in *S. griseus* and, since Southern analysis showed the *ssg*A gene to be present in *S. coelicolor* and *S. lividans*, other streptomycetes as well. The *ssg*A gene may have applied interest for the antibiotic industry. Filamentous growth of actinomycetes can be a problem in antibiotic fermentations. The possibility exists that the *ssg*A gene transformed into production cultures would result in cell that grow better as dispersed rather than as clumped mycelial masses, thus allowing better nutrient and oxygen transport with resultant increased production of secondary metabolites.

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